MOLECULAR AND CELLULAR MECHANISMS OF PRESSURE INDUCED CA^{2+} MEDIATED IMPAIRMENTS OF THE CENTRAL NERVOUS SYSTEM

I. PI: Yoram Grossman PhD **Co-PI** Gideon Gradwohl

Organization: Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

ONR award number: N000014-10-1-0163

General Background

Short term high pressure neurological syndrome (HPNS) is caused by increased ambient (hydrostatic) pressure above 1 MPa (for humans).

HPNS involves impairment of cognitive functions, autonomic nervous system alterations, sensory disturbances, motor difficulties, and general increase of brain excitability that may end up with convulsions and seizures.

Long term deterioration in health and quality of life is suspected for deep sea professional divers manifested mainly as motor difficulties and forgetfulness.

Depressed Ca^{2+}-dependent presynaptic neurotransmitter release and increased glutamate N-methyl Di-aspartate receptors (NMDARs) synaptic activity were implicated in HPNS.

Various voltage dependent Ca^{2+} channels (VDCCs) are involved with synaptic release and cellular (dendritic, somatic) integration of electrical signals.

Many NMDAR subtypes exist in the CNS. Large depolarization and excessive influx of Ca^{2+} through some of these receptors may cause cellular and network hyperexcitability and excitotoxicity.

II. Scientific and Technical Objectives

1. Characterize and model (mathematical) Ca^{2+}-dependent AMPA and NMDA synaptic potentials of CNS neurons in rat brain slices at hyperbaric pressure (HP).
2. Characterize, analyze, and model (computer simulation) Ca^{2+} currents (channels) in frog motor nerve presynaptic terminals at HP.
3. Reveal HP effects on specific VDCCs expressed in frog oocytes.
4. Study HP modulations of glutamate NMDAR subtypes consist of various subunits combinations, expressed in frog oocytes. This will be assisted by bioinformatics analysis and 3D structure modelling of specific proteins' domain(s).
5. Explore the possibility of Ca^{2+} imaging of cell cultured neurons at HP (This objective was not listed in the original proposal).

Long term Goals:

1. Suggest molecular models for the differential HP effects on various proteins, in order to reveal the molecular basis for HP sensitivity.
2. Provide a rationale for eliminating the short term HPNS and suspected long term consequences of exposure to HP.

III. Approach

1. Synaptic field potentials recordings from rat brain slices of the dentate gyrus and hippocampus CA1 region are used to study CNS AMPA and NMDA synaptic potentials response to HP obtained by helium compression in a pressure chamber. The slices are constantly perfused with physiological solution by means of high pressure pump. Mathematical modeling is applied to quantify the behavior of various synaptic vesicles releasing pools.

2. Macropatch voltage clamp current recording under the perineurial sheath of the frog motor nerve terminals are used to examine presynaptic Ca^{2+} and Na^{+} currents that are responsible for synaptic release at HP obtained by helium compression. The preparations are constantly perfused with physiological solution by means of high pressure pump. Currents are evoked by either remote nerve stimulation, or direct depolarization of the terminals. The Computer simulations of the currents are carried out by using the software NEURON platform.

3. cRNAs of specific subunits α1+ β2 + α2δ, necessary to form active VDCCs are pressure micro-injected to *Xenopus laevis* oocytes and are co-expressed after 4-5 days. VDCCs currents are measured by two-electrode voltage clamp in a pressure chamber compressed with helium at 0.1-10.1 MPa. Oocytes are constantly perfused with physiological solution by means of high pressure pump. Voltage dependent responses are tested at holding potentials with 10 mV increment voltage steps.

4. cRNA injection, proteins expression, and current measurements of NMDARs combinations are similar to those employed for VDCCs (3). Eight subunits of GluN1-1a- 4a, GluN1b- 4b and four subunits of GluN2A- D are used to express various combinations of the receptor subtypes. NMDARs are activated by 20 s application of glutamate and co-agonist glycine to the bath by the high pressure perfusion system. The bioinformatics analysis and attempted 3D structure modeling are performed by using open data banks and web-available sophisticated analysis tools such as single and multiple sequences alignment, homology modeling, and docking methods.

5. Primary cultures of rat cortical neurons (3-4 weeks old) are loaded with the Ca^{2+} indicator fluorescence dye Fura2 and ratiometric light intensity measurements are performed by fast camera mounted on a Nikon microscope at a pressure chamber compressed with helium. The cultures are constantly perfused with physiological solution by means of high pressure pump. These preliminary experiments are performed in collaboration with Prof J. Dean and Dr D. D'Agostino at the Hyperbaric Physiology Research Laboratory facility of USF, Tampa, FL.

IV. Concise Accomplishments

1. CNS synaptic potentials

Project A: Dentate gyrus synaptic residual releasable pool (RRP) response to frequency stimulation and various $[Ca^{2+}]_o$ under control and HP conditions. This provides indirect evidence for differential pressure effect on Ca^{2+} channels in CNS presynaptic terminals and readjustment of vesicular RRP.

Project B: HP modulation of hippocampal efficacy of $[Mg^{2+}]_o$ (physiological) and $[AP5]_o$ (pharmacological) blocking of NMDA synaptic response. HP considerably reduced the efficacy of the blockers, thus providing additional mechanism for pressure over-activation of NMDAR.

2. Ca^{2+} currents in nerve terminals

Experimental results in nerve terminals, corroborated by improved computer simulations, demonstrated for the first time a differential HP depression of L-type and N-type (highly sensitive) Ca^{2+} currents responsible for synaptic release. A decrease in action potential Na^+ current indirectly contributes considerably to this depression.

3. VDCCs

four types of VDCCs were tested at HP. The cellular L-type channel ($Ca_V1.2$) activity was increased while T-type channel ($Ca_V3.2$) was decreased. The presynaptic N-type channel ($Ca_V2.1$) was unexpectedly increased, but in the presence of additional control subunit (delta) depression was observed. PQ-type channel ($Ca_V2.2$) was found insensitive to HP.

4. NMDARs

We studied eight combinations of GluN1-1b, -1a + GluN2A, B, C, D has been completed. Pressure exhibits selective and differential effects on the maximal current amplitude of the receptors. GluN1-1a + GluN2A subtype is greatly potentiated while GluN1-1b + GluN2A presented dichotomic response to HP: both increase and decrease in receptor current were observed at HP. Therefore we further studied six pairs of GluN1 subunit: GluN1-2a,-2b, GluN1-3a,-3b, and GluN1-4a,-4b + GluN2B. All subtypes responded with differential degree of increase currents at HP. Bioinformatics 3D model for NTD domain of the GluN1-1a GluN1-1b subunits were constructed in an attempt to reveal HP target area.

5. Ca^{2+} imaging

Ca^{2+} imaging at pressure in rat cortical neuronal primary cultures at HP is proven feasible. Preliminary data suggest that electrical stimulus- induced cytosolic $[Ca^{2+}]$ elevation is facilitated even at low HP.

V. Expanded accomplishment

1. CNS synaptic potentials

***Project A**:* The results are described in our paper: Talpalar AE, Giugliano M, Grossman Y. Enduring medial perforant path short-term synaptic depression at high pressure. Frontiers in Cellular Neurosci, 2010 (# 3 in the Productivity list). Figures are not shown since a PDF copy of the paper is attached.

The medial perforant path (MPP), connecting entorhinal cortex with the hippocampal formation, displays synaptic frequency-dependent-depression (FDD) under normal conditions. Synaptic FDD is essential for specific functions of various neuronal networks. We used rat cortico-hippocampal slices and computer modeling for studying the effects of pressure and its interaction with $[Ca^{2+}]_o$ on FDD at the MPP synapses on granule cells. At atmospheric pressure, high $[Ca^{2+}]_o$ (4-6 mM) saturated single MPP field excitatory post synaptic potential (fEPSP) and increased FDD in response to short trains at 50 Hz. HP (10.1 MPa) depressed single fEPSPs by 50 %. Increasing $[Ca^{2+}]_o$ to 4 mM at HP saturated synaptic response at a subnormal level (only 20 % recovery of single fEPSPs), but generated a FDD similar to atmospheric pressure. Mathematical model analysis of the fractions of synaptic resources used by each fEPSP during trains (normalized to their maximum) and the total fraction utilized within a train indicates that HP depresses synaptic activity also by reducing synaptic resources. These results support the hypothesis that portions of the synaptic RRP behave like separate synaptic modules that are dependent on Ca^{2+}-entry through different channels. The utility of these module RRPs during stimulation at frequency can be impaired by HP, but may be redistributed in a Ca^{2+}-dependent manner i.e. the less affected channel will carry synaptic transmission to its maximal capacity when $[Ca^{2+}]_o$ is elevated. These frequency-dependent phenomena may explain some conserved performance at HP, and also pathological features of HPNS that may depend on environmental stimuli inducing high-frequency activity. These may be involved with diving mammals (whales, seals) adaptation to depths, and may even shed light on the problem of their susceptibility to underwater noise and sonar activity.

***Project B**:* The results are described in our paper: Mor A, Grossman Y. The efficacy of physiological and pharmacological NMDA receptor block is greatly reduced under

hyperbaric conditions. Neurosci. 169: 1-7, 2010 (# 2 in the Productivity list). Figures are not shown since a PDF copy of the paper is attached.

HPNS involves CNS hyperexcitability that is partially attributed to augmented responses of the glutamatergic NMDAR. NMDAR is blocked physiologically by Mg^{2+} and pharmacologically by DL-2-Amino-5-phosphonopentanoic acid (AP5). We have previously reported that HP augments rat hippocampus NMDAR synaptic response and generates hyperexcitability. We therefore tested HP effects on the blockade efficacy of Mg^{2+} and AP5. At HP more than double $[Mg^{2+}]_o$ and $[AP5]_o$ were needed to achieve similar effects on NMDAR synaptic response's amplitude, decay time, and time integral comparable to control conditions. $[Mg^{2+}]_o$ and $[AP5]_o$ concentration-response curves and the concentration for 50% responses' inhibition (IC_{50}s) showed similar normalized pattern at control and pressure for each parameter. The reduction in NMDAR blockers efficacy at HP may be associated with the receptor conformational change(s). This provides additional mechanism for pressure over-activation of NMDAR. Taken together with our previous studies, HP modification of NMDAR activity significantly contributes to CNS hyperexcitability. This study provides further evidence and additional mechanism for pressure augmentation of NMDAR response. This augmentation results in enhanced fEPSPs involved in short term HPNS, and excessive Na^+ and Ca^{2+} influxes that may lead to long term excitotoxic neuronal injury in the CNS.

2. Ca^{2+} currents in nerve terminals

The results are described in our paper: Aviner, B*., Gradwohl, G.*, Moore J H. and Grossman Y. Modulation of presynaptic Ca^{2+} currents in frog motor nerve terminals by high pressure. Eur J Neurosci 38(5): 2716-2729, 2013 (# 5 in the Productivity list). Figures are not shown since a PDF copy of the paper is attached**.**

Presynaptic Ca^{2+}-dependent mechanisms have already been implicated in HP depression of evoked synaptic transmission. Therefore, pressure effects on presynaptic Ca^{2+} currents were studied in frog peripheral motor nerve terminals. The combination of pharmacological, $[Ca^{2+}]_o$, voltage dependent Ca^{2+} channel (VDCC) blockers treatments, and HP perturbations revealed two components of presynaptic Ca^{2+} currents: an early fast phase (I_{CaF}), possibly carried by N type ($Ca_V2.2$) Ca^{2+} channels, and a late slow and long lasting phase (I_{CaS}) , possibly mediated by one of the L type (Ca_V1) Ca^{2+} channels. HP may have slightly shifted the voltage

dependence, reduced the amplitude and decreased the maximum (saturation level) of the Ca^{2+} currents, the I_{CaF} being more sensitive to pressure. Our recent analysis of the direct stimulation of naïve and TTX-treated nerves and the simulations of the general changes in I_{Ca} under these conditions strongly indicate that HP directly depressed the currents through Ca^{2+} channels. However, they also suggest that the natural action potential (AP) plays an important role in inducing terminal inward I_{Ca} and thus it is part of the mechanism of pressure effect. In naïve nerve, HP reduced both currents. On the other hand, in the absence of AP (I_{Na} =0 in the simulation) the relative pressure depression of the I_{CaF} (evoked by direct stimulation) was only 31% (compared to 45% at naïve terminals) while I_{CaS} was not affected! It seems that HP effect on terminal I_{CaF} is a combined depression of the AP and the channels themselves, while the I_{CaS} channels seem resistant to pressure, most of the effect being mediated by the AP depression. The differential effects of HP on the terminal currents support the idea of co-localization of at least two types of VDCCs at the presynaptic terminals which generate two physiologically different Ca^{2+} currents. The complex effect of pressure on these Ca^{2+} currents, i.e. reduction in amplitude, decrease in maximal (saturated) current and possible shift in voltage dependency, in addition to diminution of Na^{+} action potential, are the major cause of pressure depression of synaptic transmission.

3. VDCCs

Cellular channels: The results for the L-type and T-type Ca^{2+} channels are described in our paper: Aviner, B, Mor, M, Levy, S, Gradwohl, G and Grossman, Y. Selective modulation of cellular voltage dependent calcium channels by hyperbaric pressure - a suggested HPNS partial mechanism (Under revision for Frontiers in Cellular Neuroscience) (# 6 in the Productivity list). Figures are not shown since a PDF copy of the paper is attached**.**

HP selectively affects different types of cellular VDCCs: $Ca_V1.2$ (L-type) current is augmented and $Ca_V3.2$ (T-type) current is depressed. The change in current was associated with a corresponding change in channel conductance.

For the $Ca_V1.2$ channel inactivation was stronger at V_{Imax} , but was weakened at stronger depolarization. For the $Ca_V3.2$ channel, the inactivation was stronger around the threshold voltage (ΔV -20 – -10 mV) at HP, but was weakened at V_{Imax} at lower HP (1.1 MPa).

The kinetics of the channels is also modulated by HP. We measured various current parameters such as the time to peak (TTP), time constants for activation, fast and slow decay, and deactivation ('tail current'). The responses are quite complex and are voltage dependent. The results are qualitatively summarized in table 3.1:

	I_{max}	Conductance	Inactivation	TTP	τ_{Rise}	$\tau_{Decay\ Fast}$	$\tau_{Decay\ Slow}$	τ_{Tail}
$Ca_V1.2$	↑	↑	↑ (↓)	↑	↑	↓ (=)	=	↓
$Ca_V3.2$	↓	↓	↑ (=)	↓	N.A.	↓	= (↓)	N.A.

Table 3.1: General qualitative effect of HP on channel characteristics. ↑- increase, ↓- decrease, = - no change, ( ) - stronger depolarization.

HP affected the behavior of both $Ca_V1.2$ and $Ca_V3.2$, whether throughout their membrane potential activity range (maximal current, conductance, τ_{Tail} at 5.0 MPa), or just at a confined voltage range (τ_{Rise}, TTP, inactivation, $\tau_{Decay\ Fast}$). The HP effect on the two channels was generally opposite (maximal currents, conductance, TTP), but some kinetic traits shared the same HP-induced tendencies (inactivation at V_{Imax}, $\tau_{Decay\ Fast}$). Some of the effects may indicate a transient nature (conductance and partially τ_{Tail}), while other suggested that the HP effect can be reversible (mostly for $Ca_V1.2$; $\tau_{Decay\ Fast}$, τ_{Tail}, and partially also maximal current and τ_{Rise}).

These changes in the response to depolarization, both in magnitude and kinetics, would undoubtedly influence the channels functionality in neurons. Decrements in locomotion, myoclonus, tremor, changes in EEG and sleep disorders, are all HPNS manifestations that may result from these HP-induced changes.

Presynaptic channels: To further investigate the role of VDCCs in HPNS mechanisms we have completed studies on currents of the presynaptic channels $Ca_V2.1$ (P/Q-type) and $Ca_V2.2$ (N-type).

The statistical up to date data of all VDCCs are shown in table 3.2. The $Ca_V2.1$ channel is pressure resistance; the kinetics of activation and inactivation, voltage sensitivity, and the maximal current amplitude are not significantly affected (Fig 3.1A, and Table 3.2). Unexpectedly, the $Ca_V2.2$ (N-type) current ("3 subunits channel") was greatly increased at HP (Fig 3.1B, and Table 3.2). This result is surprising, since previous studies in lobster neuromuscular junction (Grossman, Colton et al. 1991)

and guinea pig cerebellar parallel fibers (Etzion and Grossman 2000) using specific blockers of the channel suggested a HP induced current depression in this channel. This apparent inconsistency may be explained by the mixed-species (human-rat-rabbit) recombinant channel expression in our study vs. 'natural' channels in the *in situ* experiments tissue, or the lack of expression of another subunit which may not be crucial for the channels' normal function, but does regulate it in a HP sensitive mechanism? Indeed, addition of the control subunit (delta) to the recombinant channel ('4 subunits channel') eliminated the HP induced increase and renders it even to depression at specific pressure (Fig 3.1C and table 3.2). Analysis of the data is still in progress.

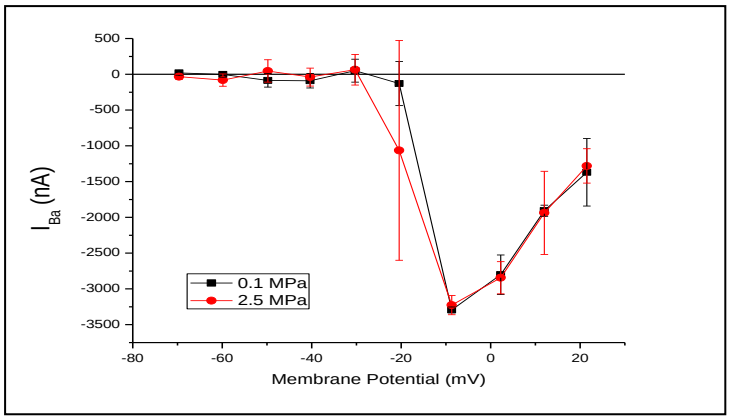
In order to verify the differential effect of HP on T-type channels, we have recently commenced experiments with $Ca_V3.1$ channels (see preliminary results in table 3.2).

In addition to the HPNS manifestations (mentioned above), several physiological VDCCs' dependent processes that are modifies by HP such as reduction in synaptic transmission due to impaired release, change in vesicular releasing pool availability, 'dendritic boosting', frequency modulation of neuronal firing, and increased muscle twitch force may well be explained by our results.

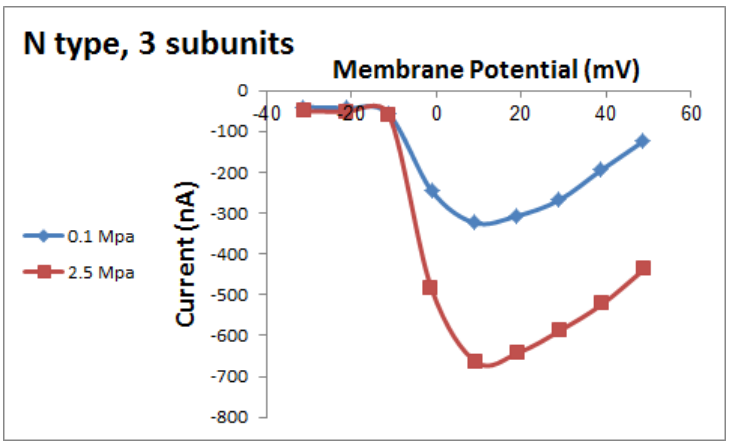
Channel	0.6MPa	1.1MPa	2.6MPa	5.1MPa
L-type $Ca_V1.2$	+22% n=16 **		+41% n=8 **	+58% n=7 *
T-type $Ca_V3.2$	-45% n=9 **	-60% n=4 *	-58% n=6 *	-51% n=6 p=0.07
T-type $Ca_V3.1$	-17% n=1 NA	+3% n=1 NA	+34% n=1 NA	+24% n=1 NA
P/Q- type $Ca_V2.1$	+21% n=1 NA	-8.5% n=2 p=0.16	-5% n=8 p=0.16	-16% n=1 NA
N-type $Ca_V2.2$ (3 S. Units)	+138% n=8 *	+168% n=3 p=0.24	+141% n=8 *	+189% n=5 p=0.056
N-type $Ca_V2.2$ (4 S. Units)	+7% n=5 p=0.83		-5% n=10 p=0.75	+15% n=6 p=0.58

Table 3.2: Statistical analysis of current amplitude modulation by HP in all VDCCs tested. * $p<0.05$, ** $p<0.01$

A P/Q type



B



C

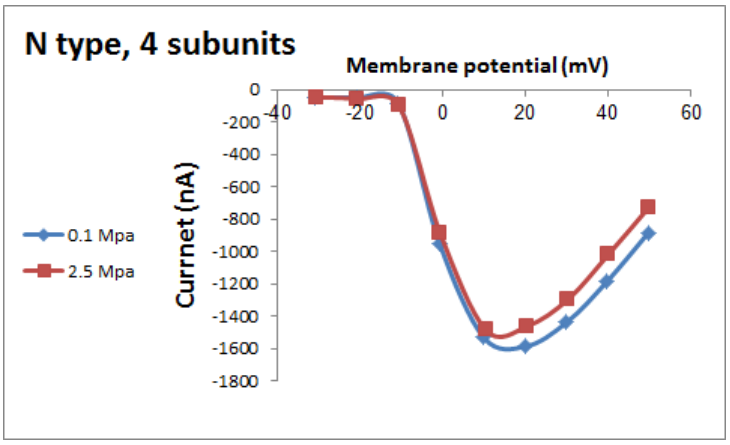


Fig 3.1: VDCCs: A, HP (2.5MPa) did not affect the I-V curve of P/Q type channels (n=8). B, HP (2.5MPa) increased maximal current in I-V curve of N-type channel consisted of 3 subunits (single oocyte). C, HP decreased max current in I-V curve of N-type channel consisted of 4 subunits (single oocyte).

4. NMDARs

GluN2 subunits: The results for GluN2 subunits (+ GluN1a,1b) are described in our paper Mor, A, Kuttner, Y, Levy, S, Mor, M, Hollmann, M and Grossman, Y. Pressure-

selective modulation of NMDA receptor subtypes may reflect 3D structural differences. Frontiers in Cellular Neuroscience 6: 1-7, 2012. (# 4 in the Productivity list). Figures are not shown since a PDF copy of the paper is attached**.**

HP (10.1 MPa) differentially affects eight specific NMDAR subtypes, GluN1(1a or 1b) co-expressed with one of the four GluN2(A-D) subunits. The statistical analysis of the results is shown in table 4.1. GluN1-1a + GluN2C, GluN1-1b + GluN2C, GluN1-1b + GluN2A and GluN1-1b + GluN2D subtypes' currents were depressed by HP. GluN1-1a + GluN2B, GluN1-1a + GluN2D and GluN1-1b + GluN2B, appeared to be 'pressure-resistant'. HP augmented *only* GluN1-1a + GluN2A current in average, although in a few oocytes the current was slightly increased (Table 4.1 highlighted).

Subunits composition	**Amplitude (nA) Mean ± SE** **0.1Mpa**	**Amplitude (nA) Mean ± SE** **10.1 MPa**	**Amplitude (%Δ) Mean ± SE** **10.1 / 0.1MPa**	**n**	**p**
GluN1-1a + GluN2A	648 ± 141	925 ± 232	+37.9± 13.7	11	0.04
GluN1-1b + GluN2A	1858 ± 189	1561 ± 201	-18.5 ± 5.5	9	0.003
GluN1-1a + GluN2B	571 ± 96	593 ± 116	-1.4 ± 7.2	12	0.6
GluN1-1b + GluN2B	561 ± 149	508 ± 144	-6.5 ± 6.4	10	0.115
GluN1-1a+ GluN2C	233 ± 31	169 ± 21	-25.0 ± 5.9	7	0.017
GluN1-1b + GluN2C	116 ± 16	61 ± 11	-47.4 ± 5.1	8	<0.001
GluN1-1a + GluN2D	63 ± 11.1	64 ± 9.3	+9.0 ± 11.6	9	0.703
GluN1-1b + GluN2D	232 ± 57	152 ± 38	-24.2 ± 7.0	9	0.017

Table 4.1: Statistical analysis of the HP response of NMDAR combinations of GluN2. Note that in GluN1-1a + GluN2A current is increased whereas in GluN1-1b + GluN2A it is decreased (highlighted).

As noted above the averaged response of its paired combination GluN1-1b + GluN2A is depressed. This 'pair' was the target of our modeling the structure of the proteins N-terminus domain (NTD). *Sequence alignment, Homology structure modeling, and Docking* methods were used to create the 3D theoretical models in order to reveal specific receptor domains which may explain the HP selectivity. The

only difference between the two combinations is a 21 amino acids outer loop present in the GluN1-1b subunit and absent in the GluN1-1a subunit. These preliminary results demonstrate that NMDAR subtypes' are differentially responding to HP, but the outer loop significance in determining the HP response is not yet clear.

GluN1 subunits: The question of GluN1 subunits role in HP response was tested in another set of experiments in which we examined all eight splice variants using newly synthesized cRNAs of: GluN1-1a -1b, GluN1-2a -2b, GluN1-3a -3b, GluN1-3a -3b, and GluN1-4a -4b, coexpressed with GluN2A. The results are summarized in table 4.2. All GluN1 variants exhibit increased current under HP conditions (see examples of responses in Fig 4.1). However, a considerable number of GluN1-4a and 4b oocytes exhibited reduced current at HP, i.e. a dichotomic response (data in red). At present we do not have an explanation for this behavior, which occurred also for the GluN1-1b in the previous set of experiments (see above).

In general the current augmentation at HP was associated with increased conductance of the channel at the maximal open state, while decreased current was associated with reduced conductance. However, this conductance could be responsible for only about 70% of the amplitude change. Additional mechanisms should be considered.

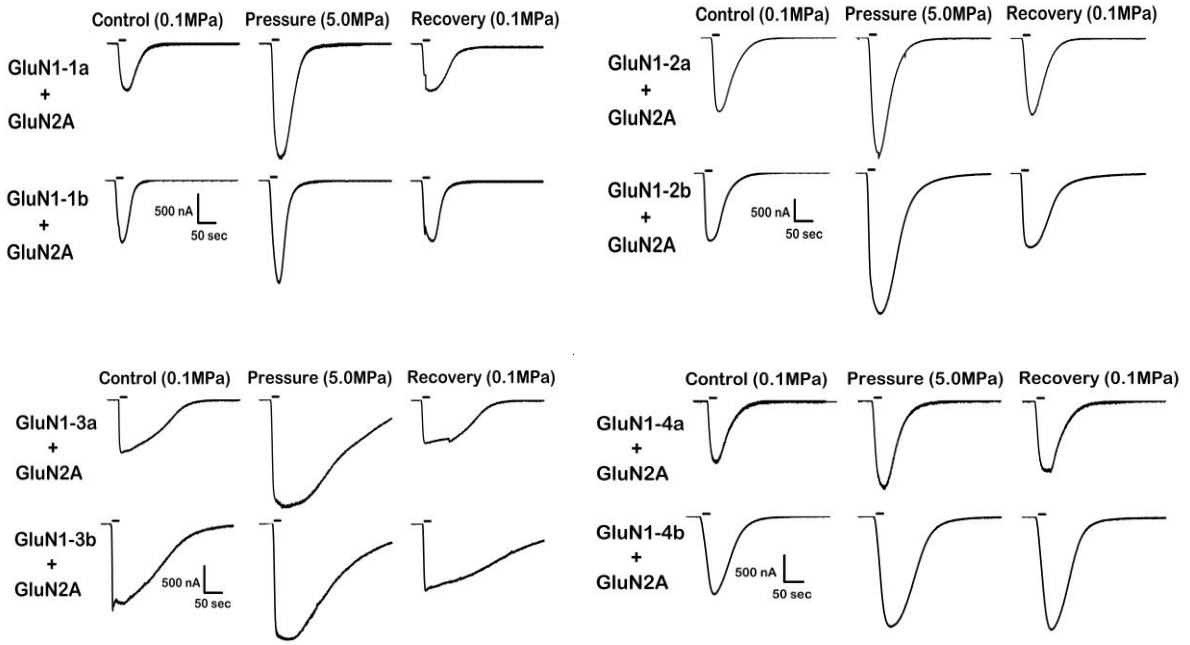


Fig 4.1: Examples of Pressure effects on 8 splice variants of GluN1 co-expressed with GluN2A. Note HP (5.0 MPa) increased the maximal current in all samples. Recovery is satisfactory excluding GluN1-4b.

Subunit composition	Amplitude(nA) Mean ± SEM 0.1MPa	Amplitude(nA) Mean ± SEM 5.1MPa	Amplitude(%Δ) Mean ± SEM	n	p
GluN1-1a + GluN2A	1542.57±251.29	2486.42±468.96	62.47±10.31	7	0.01
	0.1MPa	**10.1MPa**	**%**		
	2250.63±310.02	3813.44±594.37	71.70±17.38	4	0.032
GluN1-1b + GluN2A	1632.67±237.82	2030.06±211.57	25.16±3.91	9	<0.001
	0.1MPa	**10.1MPa**	**%**		
	2226.88±256.11	3424.04±410.31	52.51±9.07	9	<0.001
GluN1-2a + GluN2A	2018.06±244.72	2837.51±272.62	44.99±10.74	7	<0.001
GluN1-2b + GluN2A	2624.70±244.96	3347.43±213.25	32.08±8.06	10	<0.001
GluN1-3a + GluN2A	2531.63±181.885	3167.02±209.88	27.65±6.11	10	<0.001
GluN1-3b + GluN2A	1776.89±159.19	2465.71±118.28	41.89±7.44	6	<0.001
GluN1-4a + GluN2A	1075±177.17	1346.37±226.96	26.58±4.87	7	0.003
	1214.76±113.18	979.76±172.83	-19.51±10.78	3	0.212
GluN1-4b +GluN2A	1512.79±233.32	2044.67±301.41	47.38±13.30	9	<0.001
	3878.14±240.86	2345.67±322.81	-38.76±12.13	2	0.224

Table 4.2: Statistical analysis of the HP response of NMDARs with GluN1 splice variants co-expressed with GluN2A. Responses to 10.1 MPa HP are highlighted. Note the dichotomic responses of GluN1-4a and 4b which exhibit also a considerable number of oocytes in which HP reduced the maximal current amplitude (red numbers).

It is known that the transmembrane pore domain in all GluN1 and GluN2 subunits is quite conserved. Therefore, the differential response to HP is probably depends on other protein domains such as NTD or the intracellular C-terminus domain (CTD). However, it seems that the hypothesis raised in Mor et al. 2012 that the presence (in "b" variants) and the absence (in "a" variants) of the 21 amino acids outer loop in the GluN1 NTD determines the nature of the HP response ("a" - increase and "b" – decrease respectively) can not be supported.

Because of the diversity of NMDARs behavior at HP, the specific response, membrane density, and spatial distribution of a particular subtype in the mammalian CNS neurons, may lead to selective HP effects on different brain regions or even specific group(s) of neurons.

These discoveries may call for reconsideration of safety diving guidelines.

5. Ca^{2+} imaging

This project was not a part of the original award proposal. However, as the opportunity for this state of the art research has immerged when the confocal/Ca^{2+} imaging system at pressure has been completed Sep 2011 at the Hyperbaric Biomedical Research Laboratory at USF, Tampa, FL, Prof J. Dean and Dr. D. D'Agostino have agreed to collaborate on this research. The new experimental setup is shown in Fig 5.1A. Since this is the first time that such an experiment is performed, the techniques are described in more details. We incorporated a system for strict temperature control (measured at the experimental bath), continuous flow of solutions using high pressure pump, and stimulation electrodes (See Fig 5.1B).

A B

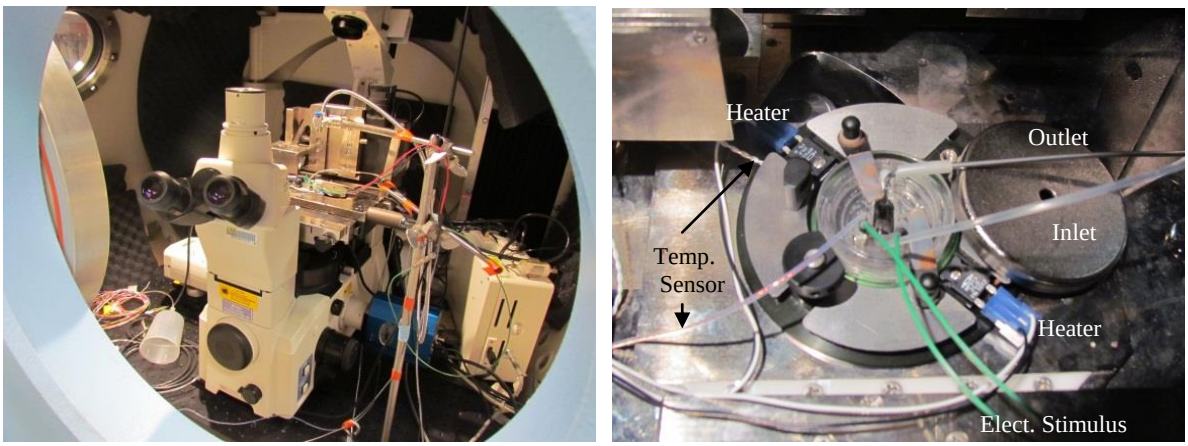


Fig 5.1: Experimental setup for Ca^{2+} imaging. A general view through the port window of the setup inside the Nautilus pressure chamber (up to 0.6 MPa). B, Experimental dish for the primary tissue culture. The system includes bath temp monitoring, feedback controlled base temperature, controlled inflow solution temperature, inlet (high pressure pump) and outlet (suction) tubing for continuous flow of solutions, and electrical stimulating electrodes.

Rat cortical neurons primary cultures were incubated for 19-30 day in aCSF at 37ºC. Prior to the experiment, each culture (on a glass cover slip) was incubated for 1 hour with 10 μM Fura2-AM and washed thoroughly thereafter (see Fig 5.3A). A dual 340 + 380 nm excitation with a Nikon microscope was perform by a cool-snap camera at 2 frames/s before and after electrical field stimulation was delivered to the bath (5 pulses train, 50 Hz, 5-10 ms duration, 10-30 V). Dynamic cytosolic [Ca^{2+}] were

acquired by computerized (Nikon Elements software) ratiometric 340/380 measurements of the Ca^{2+} indicator dye fluorescence from approximately 20 somata and 20 dendrites – selected regions of interest (ROIs) in each culture. (Fig 5.2A).

Compression by helium to 0.3 and 0.6 MPa was performed at 0.04 MPa / min. During compression the whole chamber was cooled by internal radiator in order to minimize adiabatic temperature changes. Measurements were taken at 22±0.5°C.
In preliminary control experiments pressure at this range had no significant effect on the Fura2 emission in aCSF.

A B

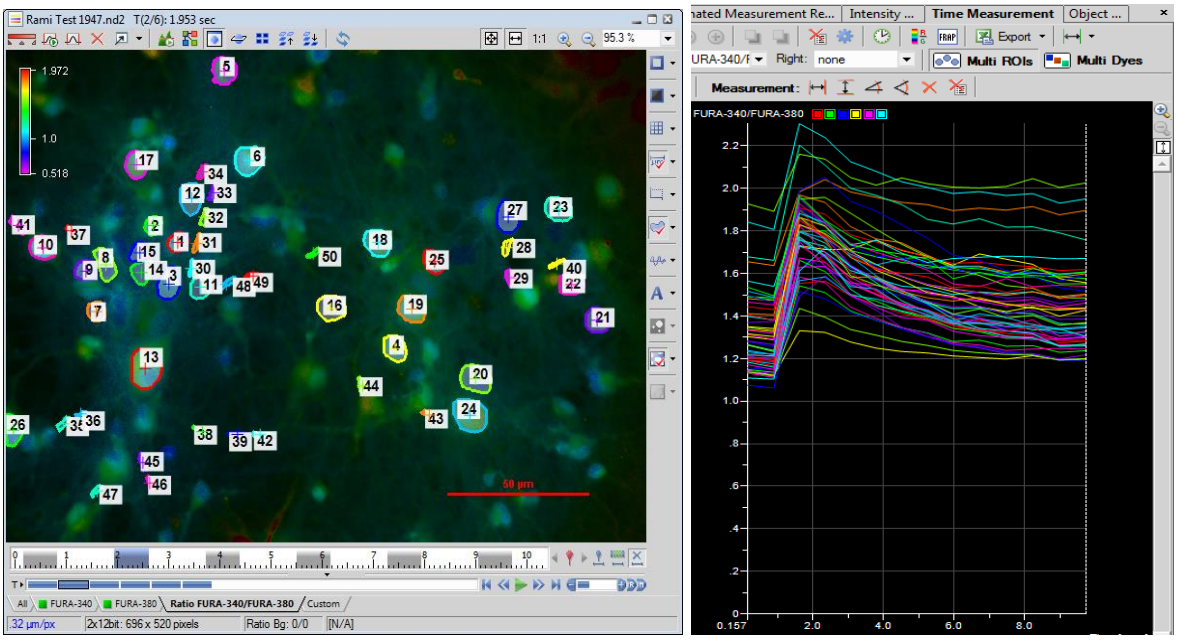


Fig 5.2: A, Selection of ROIs: Neuronal culture dyed with Fura 2-AM fluorescent (excited with 340/380 nM light) with superimposed manually selected ROIs. B. Presentation of the ratio of intensities as a function of time (s) in all ROIs selected, after electrical stimulation delivered at 1s. Note also the variability in resting level among different neurons and cell segments.

The time course of the change in cytosolic [Ca^{2+}] following an electrical stimulation at normobaric pressure is shown in Fig 5.2B. The immediate maximal rise (increased 340/380 ratio) was observed after about 0.5 s. The decay time lasted normally 8-15 s. However, in some cells recovery was much slower (see Fig 5.3 for color-coded images). The actual time course of the color coded [Ca^{2+}] measurement can be seen in the supplemented video clip (Rami Test 1944.avi).

The maximal rise was augmented already at 0.3 MPa (20 msw!) by 25±5% in somata, and by 22±5% in dendrites (n=9, $p<0.01$). This augmentation subsided after a few minutes in 7/9 cultures and reached a steady state of about 10% at 20-25 minutes of sustained pressure. The cytosolic [Ca^{2+}] baseline seemed to be elevated at HP by variable degree in both dendrites and somata with poor recovery upon decompression. Increased pressure to 0.6 MPa only slightly added to the transient increase following stimulation, but more cells responded to the stimulus under these conditions.

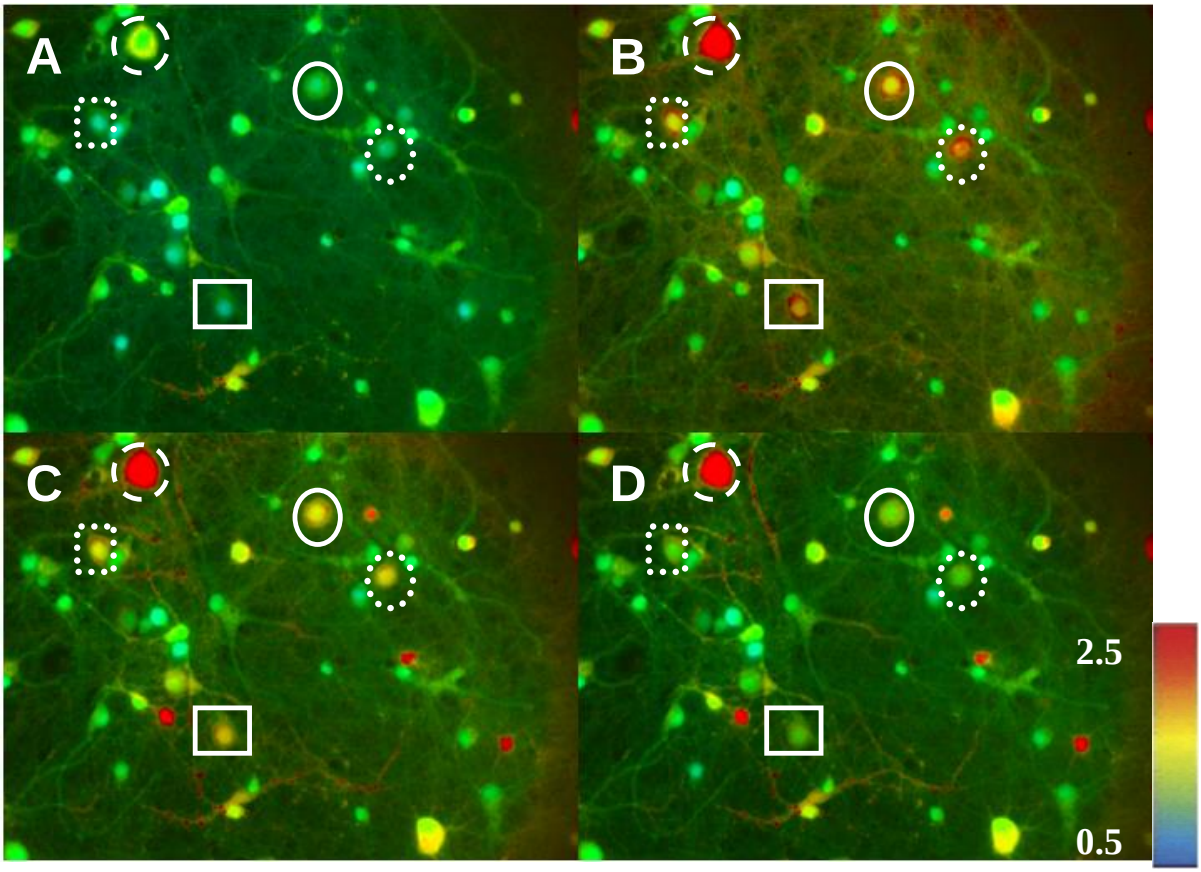


Fig 5.3: Example of culture response to electrical stimulation at normobaric pressure. Color-coded ratio 340/380 emission of 5 ROIs of somata are marked; A, pre stimulus, post stimulus: B, 1 s, C, 3 s, and D, 8 s. In some cells the decay of $[Ca^{2+}]_i$ was slower, e.g. dashed circle. Note also the increase in many unmarked dendritic branches.

The Ca^{2+} imaging at helium pressure (up to 0.6 MPa) in rat cortical neurons primary culture is proven feasible. Our preliminary data demonstrate that electrical stimulus-induced cytosolic [Ca^{2+}] rise is transient, and that it is increased at low hyperbaric pressures. Cytosolic [Ca^{2+}] baseline may also increase indicating difficulties in [Ca^{2+}] homeostasis.

The overall HP effect shown here is most probably a combination of pressure modulations of Ca^{2+} influx through several VDCCs expressed in the neurons, the most prominent one is likely to be L-type $Ca_V1.2$, which was shown by us (Aviner et al. 2014, above) to be augmented by HP. These results suggest unexpectedly high sensitivity of cortical neurons to relatively low HP exposure (at least in tissue culture). This should call for new consideration of HP risk in diving.

These preliminary results are the basis for our collaborative effort with Prof. J.B. Dean and Dr. D. D'Agostino (USF, Tampa FL) of submitting a research grant proposal to the Israel - USA Binational Science Foundation (BSF) this year.

VI. Work plan

This is a final report after NCE ended on 31.12.2013.

VII. Major Problems/Issues

None

VIII. Technology transfer

None

IX. Foreign collaboration

Dr Yossi Kuttner, BGU, Israel (3D protein structure modeling of NMDARs).

Dr Homer J Moore, U Florida, FL, USA (Various Ca^{2+} currents in frog nerve terminals and their response to HP).

Prof Michele Giugliano, Antwerp U, Belgium (Mathematical modeling of synaptic vesicles pools under HP conditions).

Dr. Adolfo Talpalar, Karolinka Inst., Sweden (CNS synapses at HP and their vesicles residual releasing pool).

Prof Michael Hollmann, Ruhr U, Bochum, Germany (NMDAR cRNAs production and analysis of the receptors HP response).

Prof Arie Moran, BGU, Israel (VDCCs cRNAs production).

Prof Daphna Atlas, HUIJ, Israel (VDCCs cRNA donation).

Prof Natan Daskal, TAU, Israel (VDCCs cRNA donation).

Prof Jay Dean, USF, Tampa, FL, USA (Ca^{2+} imaging in primary cortical culture at HP).

Dr. Dominic D'Agostino, USF, Tampa, FL, (Ca^{2+} imaging in primary cortical cultures at HP).

X. Productivity

A. Refereed Journal Articles

1. Aviner, B, Gnatek, Y, Gradwohl, G, Grossman, Y, 2010. Hyperbaric pressure effects on voltage-dependent Ca^{2+} channels: Relevance to HPNS. Undersea Hyperbar. Med. J. 37:245-258. 2010.

   Accompanied by invited 'Editorial Perspectives' by Prof Oliver Friedrich, Germany.

2. Mor, A and Grossman, Y. 2010. The efficacy of physiological and pharmacological NMDA receptor block is greatly reduced under hyperbaric conditions. Neurosci. 169: 1-7.

3. Talpalar, AE*, Giugliano, M* and Grossman, Y. 2010. Enduring medial perforant path short-term synaptic depression at high pressure. Frontiers in Cellular Neurosci. http://www.frontiersin.org/Journal/Abstract.aspx?s=156&name=cellular_neuroscience&ART_DOI=10.3389/fncel.2010.00128.

4. Mor, A, Kuttner, Y, Levy, S, Mor, M, Hollmann, M and Grossman, Y, 2012. Pressure-selective modulation of NMDA receptor subtypes may reflect 3D structural differences. Frontiers in Cellular Neuroscience 6: 1-7. http://www.frontiersin.org/Journal/Abstract.aspx?s=156&name=cellular_neuroscience&ART_DOI=10.3389/fncel.2012.00037.

5. Aviner, B*, Gradwohl, G*, Moore, J H and Grossman, Y. 2013. Modulation of presynaptic Ca^{2+} currents in frog motor nerve terminals by high pressure. Eur J Neurosci 38(5): 2716-2729.

6. Aviner, B, Mor, M, Levy, S, Gradwohl, G and Grossman, Y. 2014. Selective modulation of cellular voltage dependent calcium channels by hyperbaric pressure - a suggested HPNS partial mechanism (under revision for Frontiers in Cellular Neuroscience).

7. Aviner, B, Bliznyuk, A and Grossman, Y. 2014. Selective pressure modulation of Ca^{2+} currents of presynaptic PQ- and N-type channels expressed in frog oocytes (In preparation).

8. Aviner, B, Ari C, D'Agostino, D, Dean, J and Grossman, Y. 2014. Ca^{2+} imaging of cortical neurons under hyperbaric conditions. (In preparation)

9. Bliznyuk, A, Aviner, B, Mor, A, Hollmann, M, Golan, H and Grossman, Y. 2014 The role of GluN1 subunits in pressure modulation of NMDA receptor currents. (In preparation)

B. Non-Refereed significant Publication

None

C. Books or chapters

1. Grossman, Y., Aviner, B. and Mor, A., High Pressure effects on Mammalian Central Nervous System. In, Comparative High Pressure Biology. Ed P. Sebert, Science publishers, Enfield, New Hampshire pp 161-186, 2010. (Invited)

2. Daniels, S. and Grossman, Y. Pressure effects on cells. In, Comparative High Pressure Biology. Ed P. Sebert, Science publishers, Enfield, New Hampshire pp 121-142, 2010 (Invited).

3 Amir Mor. PhD thesis: High pressure modulation of mammalian CNS NMDA receptor response, 2012 (Approved).

4. Ben Aviner PhD thesis: Hyperbaric effects on voltage dependent Ca^{2+} channels activity, 2014 (in preparation)

5. Alice Bliznyuk MSc thesis: the role of NMDA receptors in High Pressure Neurological syndrome (HPNS): a molecular approach 2014 (Approved)

D. Technical reports

None

E. Workshops and Conferences

1. Aviner B, Mor M, Grossman Y. P/Q type voltage dependent Ca^{2+} channel may resists high pressure. Israel Society for hyperbaric & diving medicine. Annual scientific meeting, Nov 2010, Tel-Aviv, Israel.

2. Aviner B, Mor M, Grossman Y. Indication for P/Q type voltage dependent Ca^{2+} channel resistance to hyperbaric pressure. Israel Society for Neuroscience (ISFN), Proceedings annual scientific meeting, Dec 2010, Eilat, Israel.

3. Mor A, Kuttner Y, Levy S, Mor M, Hollmann M, Grossman Y. High pressure differentially affects eight NMDA receptor subtype combinations. Israel Society for hyperbaric & diving Medicine (ISHDM). Annual scientific meeting, Nov 2010, Tel- Aviv, Israel.

4. Mor A, Kuttner Y, Levy S, Mor M, Hollmann M, Grossman Y. Pressure selective modulation of NMDA receptor subtypes' currents may reveal 3D structural differences. Israel Society for Neuroscience (ISFN), Proceedings annual scientific meeting Dec 2010, Eilat, Israel.

5. Aviner B, Gradwohl G, Grossman Y. Pressure modulation of presynaptic Ca^{2+} currents in frog motor nerve terminals. European Underwater Baromedical

Society (EUBS), Proceedings annual scientific meeting, Sep 2010, Istanbul, Turky.

6. Aviner B, Grossman, Y. P/Q type voltage dependent Ca^{2+} channel (CaV2.1) is resistant to hyperbaric pressure. Undersea hyperbaric medicine society (UHMS) Proceedings annual scientific meeting, June 2011, Fort Worth, TX USA.

7. Aviner B, Gradwohl G, Grossman G 2011. HPNS may be associated with differential pressure depression of presynaptic Ca^{2+} currents in nerve terminals. South pacific underwater medical society (SPUMS), Proceedings annual scientific meeting, May 2011, Guam, USA.

8. Aviner B, Grossman Y. Ca^{2+} 2.1 channel (P/Q) may sustain partial synaptic transmission at hyperbaric pressure. South pacific underwater medical society (SPUMS), Proceedings annual scientific meeting, May 2011, Guam, USA.

9. Gradwohl G, Aviner B, Grossman Y. Modulations of Ca^{2+} currents in frog presynaptic motor axons under high pressure conditions: Computer modeling. European Underwater Baromedical Society (EUBS), Proceedings annual scientific meeting, Sep 2011, Gdansk, Poland.

10. Aviner B, Mor M, Bliznyuk A, Grossman Y. Selective modulation of voltage dependent Ca^{2+} channels by HP. Israel society for hyperbaric & diving medicine (ISHDM), Annual scientific meeting, Aug 2012, Eilat, Israel.

11. Aviner B, D'Agostino D, Dean J, Grossman Y. Augmented Ca^{2+} influx in cultured rat neurons at HP, IHPBG Bi-annial scientific meeting Aug 2012 , Eilat, Israel.

12. Bliznyuk A, Mor A, Aviner B, Hollmann M Grossman Y. Hyperbaric pressure effects on NMDA receptor variants. 'Tricontinental' joint scientific meeting (EUBS, SPUMS & SAUHMA) on diving and hyperbaric medicine, Sep 2013, Reunion Island, France.

13. Bliznyuk A, Aviner B, Hollmann M, Golan H Grossman Y. Hyperbaric pressure modulation of NMDA receptor variants. Israel Society for Neuroscience (ISFN), Proceedings annual scientific meeting, Dec 2013, Eilat, Israel.

14. Aviner B, D'Agostino D, Dean J, Grossman Y. Augmented Ca^{2+} influx in cultured rat neurons at hyperbaric pressure. 'Tricontinental' joint scientific meeting (EUBS, SPUMS & SAUHMA) on diving and hyperbaric medicine, Sep 2013, Reunion Island, France.

15. Bliznyuk A, Aviner B, Hollmann M, Golan H Grossman Y. Hyperbaric pressure effects on six GluN1 variants coexpressed with GluN2A subunit of the NMDA receptor. Israel society for hyperbaric & diving Medicine (ISHDM), Annual scientific meeting, Nov 2013, Tel- Aviv, Israel.

F. Patents

None

G. Awards /Honors

1. Invited seminar: Dept of anesthesia, Dept of Hyperbaric Medicine, Rigs Hospital, Copenhagen, Denmark. Title: Cellular and Molecular Aspects of Hyperbaric Pressure Modulation of NMDA Receptors Activity. Sep 2010.
2. Chairman, organizing & scientific committee for the Israel Society Hyperbaric & Diving Medicine (ISHDM), Nov 2010.
3. Invited visiting committee expert for AERES evaluation of a research group activity: Inhalopharmacologie experimentale et cliniq. Toulon, France, Mar 2011.
4. Member of the advisory scientific committee for the EUBS meeting in Gdansk, Poland, Aug 2011.
5. Eshkol Fellowship, Israeli Ministry of Science & Technology 2008-2010 for multidisciplinary research to Amir Mor and Yoram Grossman.
6. Amir Mor. 2011. Prize for excellence in biomedical research, Faculty of Health ciences, Ben-Gurion University of the Negev.
7. Amir Mor. 2011. Dean's prize for best PhD student. Faculty of Health Sciences, Ben-Gurion University of the Negev.
8. Chairman, scientific committee, for the joint Israel Society Hyperbaric & Diving Medicine (ISHDM) and the International High Pressure Biology Group (IHPBG) meeting in Eilat, Israel, Nov 2012.

XI. Award Participants

Military personnel: NA

Amir Mor, MD PhD student

Ben Aviner, MSc PhD student

Alice Bliznyuk, MSc student